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Homologous recombination-dependent initiation of DNA replication from DNA damage-inducible origins in *Escherichia coli*

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Escherichia coli **cells induced for the SOS response express inducible stable DNA replication (iSDR) as an SOS function. Initiation of iSDR is independent of transcription, translation and DnaA protein, which are essential for initiation of DNA replication from *oriC*. We found that a *recA* mutant that is defective in recombination but proficient in SOS induction could not elicit iSDR. In contrast, iSDR was enhanced by *recD* and *recJ* mutations that inactivate the exonuclease V activity of the RecBCD enzyme and the RecJ exonuclease activity, respectively. A mutation in the *ruvC* gene that blocks the resolution of recombination intermediates (i.e. Holliday structures) also enhanced iSDR. Furthermore, inhibition of branch migration by *recG* or *ruvAB* mutations dramatically increased the iSDR activity. *recBC* mutants are defective in iSDR induction but the defect was suppressed by a mutation in the *sbcA* gene. The major product of minichromosomes replicated by iSDR was covalently closed circular monomers. We propose that recombination intermediates (i.e. D-loop structures) created by the action of RecA recombinase and RecBC(D) helicase play a central role in initiation of iSDR.**

Key words: D-loop/DNA replication/RecBCD/resolvase/SOS

Introduction

Escherichia coli RecA protein, the product of the *recA* gene, promotes homologous pairing and strand exchange *in vitro* between complementary single-stranded (ss) DNA and double-stranded (ds) DNA molecules (the recombinase activity); this reaction is suggested to be a key step leading to homologous recombination *in vivo* (for reviews, see Kowalczykowski, 1991; West, 1992). In addition to RecA protein, the products of the *recB*, *recC* and *recD* genes, which constitute the RecBCD enzyme, play a central role in conjugational and transductional recombination and UV light-induced DNA damage repair (RecBCD pathway; for review, see Smith, 1988). The RecBCD enzyme is a multifunctional enzyme. In addition to a chi-sequence specific endonuclease activity (Ponticelli *et al.*, 1985; Dixon and Kowalczykowski, 1991), it contains an ATP-dependent exonuclease activity for ds and ss DNA (the Exo V activity), an ATP-stimulated endonuclease activity for ss DNA, and an ATP-dependent DNA helicase activity (for review, see Smith, 1990). The helicase activity is thought to provide a source of ss DNA for RecA protein to initiate recombination between duplex molecules (for review, see Clark, 1991). Therefore, *recB* and *recC* mutants are deficient in conjugational and transductional recombination and are sensitive to UV light. These properties of *recB* and *recC* mutants are known to be suppressed by a mutation, in the *sbcA* gene, that derepresses the expression of exonuclease VIII (Exo VIII), the *recE* gene product, and leads to the activation of the RecE pathway (for review, see Clark, 1991). Exo VIII digests one strand of ds DNA processively in the 5′ to 3′ direction, resulting in the production of ss DNA that is apparently used by the RecA protein for recombination. Thus, similar recombination intermediates are thought to be created by RecA protein in both *recBC*$^+$ *sbcA*$^+$ and *recBC sbcA* strains.

In contrast to *recB* and *recC* mutants, *recD* mutants remain proficient in recombination and are resistant to UV light (Amundsen *et al.*, 1986; Lloyd *et al.*, 1988; Lovett *et al.*, 1988). This perplexing result could be explained by the *in vitro* observation that purified RecBC enzyme (without the RecD subunit) possesses helicase activity but not Exo V activity (Palas and Kushner, 1990). Moreover, a *recBC*-dependent DNA helicase activity has recently been demonstrated *in vivo* in *recD* mutants (Rinken *et al.*, 1992).

Like *recD* mutations, *recJ* and *recN* mutations have little adverse effect, except in *recB recC sbc* strains, on conjugational recombination (Lovett *et al.*, 1988; Lloyd and Buckman, 1991b). Moreover, neither mutation confers a UV-sensitive phenotype by itself (Lloyd *et al.*, 1988; Lovett *et al.*, 1988). However, *recD recJ* and *recJ recN* double mutants were found to be slightly deficient in recombination and moderately sensitive to UV light (Lloyd and Buckman, 1991b). Furthermore, a combination of mutations in all three genes rendered cells very deficient in recombination and strikingly sensitive to UV light (Lloyd and Buckman, 1991b). These observations have led to the suggestion that these genes provide overlapping activities that compensate for one another in the single mutants. Although RecJ protein is known to have a 5′-specific ss DNA exonuclease activity (Lovett and Kolodner, 1989), the molecular mechanism of the compensation has not been elucidated.

Following homologous pairing and strand exchange, the recombination intermediates have to be resolved into recombinant DNA products by cleaving Holliday junctions. This resolution step was shown *in vitro* to be carried out by the product of the *ruvC* gene, resolvase (Connolly *et al.*, 1991; Dunderdale *et al.*, 1991; Iwasaki *et al.*, 1991). Holliday junctions may be recognized by RecG or RuvAB proteins, which catalyze branch migration (Tsaneva *et al.*, 1992; Lloyd and Sharples, 1993). The junctions would then be cleaved by RuvC protein. However, *ruvC* mutants are known to produce close to normal numbers of recombinants in conjugational and transductional recombination (Lloyd, 1991). This recombination proficiency in *ruvC* mutants can be severely decreased by introducing a *recG* mutation

(Lloyd, 1991). Thus, it is suggested that RecG protein might also be involved in the resolution of recombination intermediates *in vivo* (Lloyd and Sharples, 1993).

Chromosome replication in *E.coli* is initiated at a unique site called *oriC* (Hiraga, 1976). Every round of initiation requires transcription, protein synthesis and the initiator protein, DnaA (for review, see McMacken *et al.*, 1987). Upon induction of the SOS response, however, a different type of DNA replication is activated, which does not require transcription, protein synthesis or DnaA protein for initiation (Kogoma and Lark, 1970, 1975; Lark, 1972; Ciesla and Jonczyk, 1980; Magee *et al.*, 1992). This type of DNA replication is termed inducible stable DNA replication (iSDR). Initiation of iSDR occurs primarily in the *oriC* and *terC* regions of the chromosome (Magee *et al.*, 1992). The origin in the *oriC* region has been localized between the *Hin*cII (coordinate −179; Meijer *et al.*, 1979; Sugimoto *et al.*, 1979) and the *Xho*I (+417) sites, which includes the minimal *oriC* (Magee *et al.*, 1992).

Initiation of iSDR leads to semi-conservative replication of the entire *E.coli* genome for many hours (Kogoma and Lark, 1970). iSDR is considerably more resistant to UV irradiation than normal DNA replication (Kogoma *et al.*, 1979), and it appears to be error-prone (Lark and Lark, 1978). Except for DnaA protein, iSDR requires several gene products that are also necessary for normal DNA replication; they include DnaB, DnaC, DnaE, DnaG and DnaT (Kogoma and Lark, 1975; Masai and Arai, 1988). However, iSDR differs from normal replication in one important aspect: it requires a *recA*$^+$ function (Lark and Lark, 1978). In addition to the recombinase activity mentioned above, RecA protein mediates the self-cleavage reaction of the LexA repressor (the coprotease activity), which leads to derepression of the LexA regulon genes and induction of the SOS response (for review, see Little, 1991). It has been demonstrated that derepression of the *recA* gene and activation of amplified RecA protein are a necessary and sufficient condition for the induction of iSDR (Magee and Kogoma, 1990). No other LexA regulon genes are necessary for iSDR. Moreover, mutations in the *recB* and *recC* genes were found to block the initiation of iSDR (Magee and Kogoma, 1990).

In this study, we examined the effects of various *rec* mutations on iSDR in order to elucidate the molecular mechanism of iSDR initiation. The results obtained strongly suggest that recombination intermediates (D-loops) created by the action of RecA and RecBC(D) proteins are involved in the initiation step of iSDR.

Results

Induction of iSDR depends on the recombination activity of RecA

To determine whether or not the RecA recombinase function is involved in iSDR, we sought a *recA* mutant that was deficient in recombination but proficient in coprotease activity. Since a recombination deficient phenotype is often associated with a constitutive coprotease activity (Tessman and Peterson, 1985), *recA* mutants were first screened for chronic SOS induction and then tested for recombination proficiency. The mutants were obtained by introducing mutagenized miniF-*recA*$^+$ plasmids into a *recA* deletion strain (see Materials and methods). The miniF-*recA* plasmid is in numerical parity with the host chromosome and is stably maintained in host cells (Dutreix *et al.*, 1989). Of 37 protease-constitutive *recA* mutants isolated, 10 were found to be defective in recombination. One of them, *recA428*, was further characterized. The *recA428* gene was found to have a single base pair substitution that causes a Gly200 to Asp change in the L2 disordered loop of the RecA protein (Story and Steiz, 1992). The mutant protein is severely deficient in recombination activity (Table I).

Table I. Recombinase activity of RecA428

Allele	Relative efficiencies		
	Conjugational recombination	Intrachromosomal recombination	Plating of λ*bio*
recA$^+$	1	1	1
recA428	$<3 \times 10^{-5}$	$<4 \times 10^{-2}$	$<10^{-6}$
Δ*recA*	$<3 \times 10^{-5}$	$<4 \times 10^{-2}$	$<10^{-6}$

To determine the inducibility of iSDR by thymine starvation in *recA428* cells, a *recA* deletion strain (AQ8014) was transformed with a miniF-*recA428* plasmid (AQ8110). For a control, the same strain was transformed with a miniF-*recA730* plasmid (AQ8112). In contrast with RecA428 protein, RecA730 protein is known to possess a normal recombination activity as well as a constitutive coprotease activity (Witkin *et al.*, 1982). SOS inducibility was first determined in these *recA* strains by measuring the expression of an *sfiA*::*lacZ* operon fusion carried by a lysogenizing λ phage. As shown in Figure 1A, the SOS response is fully induced in both *recA* strains without thymine starvation, and the induction level is about twice as much as (AQ8112) or comparable to (AQ8110) that of the wild-type (wt) strain which was starved of thymine for ~100 min, a condition that is sufficient to induce iSDR. The iSDR activity of these strains was measured as follows. Cells grown to exponential phase were induced for iSDR by starving them of thymine for 100 min. A mixture of [^{3}H]thymine, rifampicin (RIF) and chloramphenicol (CAM) was added at the end of starvation (time 0), and subsequent DNA synthesis was monitored by measuring incorporation of radioactive thymine into the acid-insoluble fraction. For uninduced controls, the mixture was added without starvation. Figure 1B shows that *recA428* cells ceased DNA synthesis 1 h after the addition of RIF and CAM in both induced and uninduced cultures. In contrast, *recA730* cells continued DNA synthesis for 5 h in the induced culture despite the presence of RIF and CAM. Even uninduced *recA730* cells continued DNA synthesis albeit at a low rate. Thus, continuation of iSDR requires the recombinase activity of RecA protein. It should be noted that, although the *sfiA*::*lacZ* fusion was fully expressed in *recA730* cells without inducing treatment, thymine starvation led to an increased iSDR activity. This is consistent with the previous observation that some constitutive component(s) for iSDR is limiting in exponentially growing cells and that accumulation of the component during inducing treatments leads to increased iSDR activities (Magee and Kogoma, 1990).

Figure 1B also shows that the induced *recA428* cells synthesized more DNA within the first hour than did the uninduced cells. The difference could reflect the replication originated from *oriC* upon the addition of thymine after starvation. It has been demonstrated that the initiation

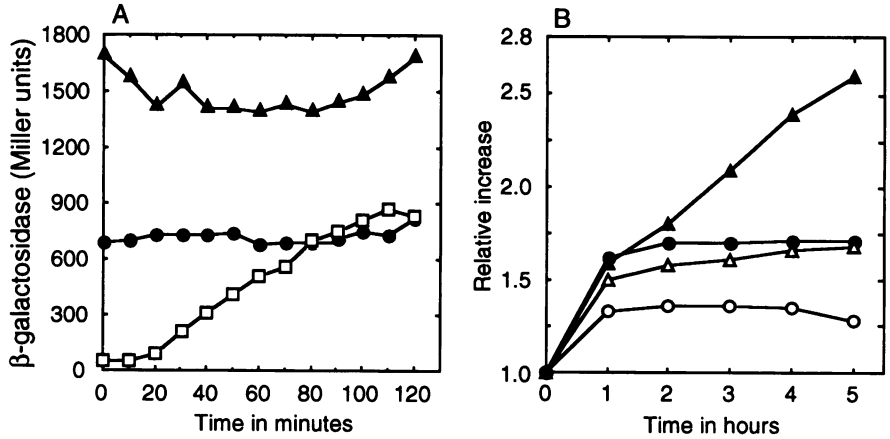

Fig. 1. Effects of *recA* mutations on iSDR. (**A**) A *recA* deletion strain (AQ8014) carrying miniF-*recA428* (AQ8110, ●), miniF-*recA730* (AQ8112, ▲) or miniF-*recA*$^+$ (AQ8115, □) plasmid was grown to 1.5 × 10^8 cells/ml in the presence of thymine. The cells were then filtered, washed and resuspended in medium lacking thymine. The cell suspensions were incubated at 37°C for 2 h. Samples were taken at the indicated times and β-galactosidase activity was measured as described by Miller (1972). (**B**) AQ8110 (●○) and AQ8112 (▲△) were grown in the presence of [^{3}H]thymine (10 μCi/8 μg/ml) to 1.5 × 10^8 cells/ml. After starving the cells of thymine for 100 min, a mixture of [^{3}H]thymine (10 μCi/8 μg/ml), RIF (200 μg/ml) and CAM (150 μg/ml) was added (filled symbols). Control cells were not starved but were immediately incubated with the mixture (open symbols). Samples were withdrawn at intervals, and radioactivity in the TCA-insoluble fraction was measured by liquid scintillation counting. Relative increase of DNA was determined as described in Materials and methods.

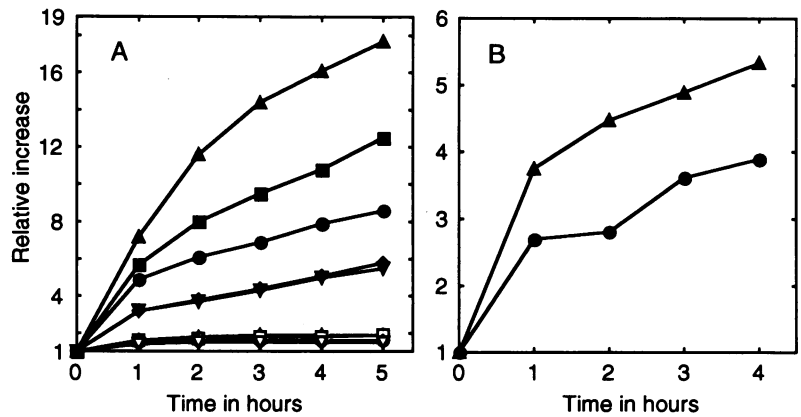

Fig. 2. Effects of *recD*, *recJ*, *recF* and *recN* mutations on iSDR and the copy number of the *oriC* region. (**A**) AQ634 (wt, ●○), AQ8134 (*recD*, ▲△), AQ3362 (*recJ*, ■□), AQ4790 (*recF*, ◆ ◇) and AQ3359 (*recN*, ▼▽) were grown in the presence of [^{3}H]thymine (10 μCi/8 μg/ml) to 2 × 10^8 cells/ml, and treated as described in the legend to Figure 1B except that cells were starved of thymine for 80 min. Filled symbols, starved; open symbols, unstarved. (**B**) AQ634 (wt, ●) and AQ8134 (*recD*, ▲) were grown in the presence of [^{3}H]thymine (5 μCi/8 μg/ml) to 1.5 × 10^8 cells/ml and starved of thymine for 80 min. After addition of thymine (8 μg/ml), RIF (200 μg/ml) and CAM (150 μg/ml), samples (0.5 ml) were withdrawn at intervals. Chromosomal DNA was prepared from each sample and the amounts of labeled DNA normalized to give equal ^{3}H counts were digested with *Hin*dIII and electrophoresed in a 1% agarose gel. Southern blot hybridization was carried out as described previously (Magee *et al.*, 1992) to determine the copy number of the *oriC* region. For the probe, a DNA fragment carrying a *gidA* sequence (Walker *et al.*, 1984) between the *Pst*I (coordinate −1560) and *Bam*HI (−100) sites was used.

potential for replication from *oriC* is accumulated during thymine starvation and that the potential can be used in the presence of CAM (Donachie *et al.*, 1968; Kogoma and Lark, 1975). Our result suggests that the potential could also be used in the presence of RIF and CAM.

Active RecBCD and RecJ exonucleases reduce the iSDR activity

Mutations in *recD* eliminate the Exo V activity of the RecBCD enzyme with a moderate effect on the helicase activity (Palas and Kushner, 1990). Figure 2A shows that the initial rate of iSDR was greatly enhanced by the *recD1903*::mini-*tet* mutation. The induced *recD* mutant cells continued DNA synthesis for a 5 h incubation period, by the end of which more than twice as much DNA was synthesized in the mutant cells as in the wt cells. The result suggests that the Exo V activity of RecBCD (as in *recD*$^+$ cells) has an inhibitory effect on iSDR, and that the ss or ds linear DNA molecule that is a substrate of the enzyme is involved in the initiation of iSDR. The difference in the amount of DNA synthesized by iSDR between the experiments shown in Figures 1B and 2A is due to a difference in the strains used. The extent to which iSDR is expressed depends on the genetic background of *E.coli* strains (see also Figures 4 and 5).

The *recJ284*::Tn*10* mutation also enhanced iSDR, although less DNA was synthesized in the *recJ* mutant than in the *recD* mutant (Figure 2A). This suggests that the exonuclease activity of RecJ protein also has an inhibitory effect on iSDR. On the other hand, the *recF332* and *recN261* mutations reduced the iSDR activity slightly (Figure 2A).

The iSDR activity in the *recD* mutant cells was further analyzed by measuring the copy number of the *oriC* region, which includes an origin for iSDR (Magee *et al.*, 1992).

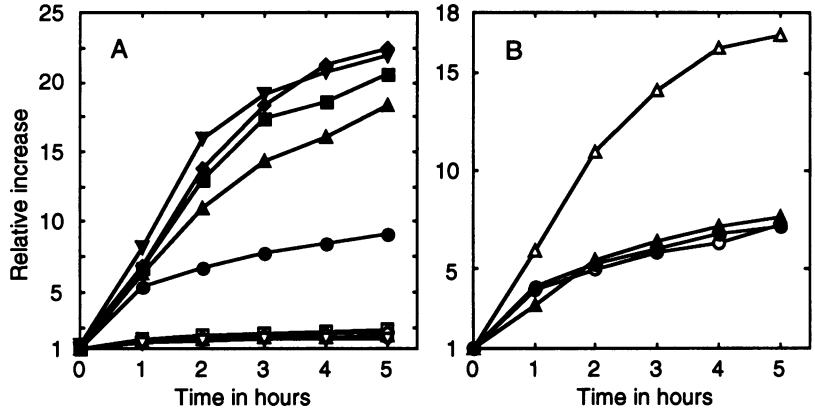

Fig. 3. Effects of *ruvC* and *recG* mutations on iSDR. (**A**) AQ634(wt, ●○), AQ7960 (*ruvC*, ▲△), AQ8130 (*recG*, ■□), AQ8208 (*recD recG*, ◆ ◇ and AQ8194 (*recJ recG*, ▼▽) were grown in the presence of [^{3}H]thymine (10 μCi/8 μg/ml) to 2 × 10^8 cells/ml and treated as described in the legend to Figure 1B except that cells were starved of thymine for 80 min. Filled symbols, starved; open symbols, unstarved. (**B**) AQ634 (wt) carrying pMZ18 (○) or pMZ18-ruvC$^+$ (●) and AQ7960 (*ruvC*) carrying pMZ18 (△) or pMZ18-ruvC$^+$ (▲) were grown in the presence of [^{3}H]thymine (10 μCi/8 μg/ml) and IPTG (1 mM) to 2 × 10^8 cells/ml, and starved of thymine for 80 min. After addition of [^{3}H]thymine, RIF and CAM, cells were treated as described in the legend to Figure 1B.

The copy number was determined by Southern blot hybridization as described previously (Magee *et al.*, 1992). Figure 2B shows that the copy number of the *oriC* region increased 5-fold in the induced *recD* mutant cells during the 4 h incubation whereas DNA increase was ~3.5-fold in the induced wt cells, indicating that initiation of iSDR at the *oriC* region was indeed enhanced in *recD* mutants. The relative increase of total DNA measured by [^{3}H]thymine incorporation (Figure 2A) was ~3 times higher than the relative copy number increase at the *oriC* region (Figure 2B) in both wt and *recD* mutant cells. This is because the total DNA synthesis measured by [^{3}H]thymine incorporation reflects not only the DNA replications originated from the *oriC* region, but also those from the *terC* region, which includes the other major origin for iSDR, and from other minor origins elsewhere (Magee *et al.*, 1992).

Resolution and migration of recombination intermediates inhibit iSDR

Figure 3A shows that the *ruvC51* mutation dramatically enhanced the rate of iSDR, suggesting that Holliday structures are involved in the initiation reaction of iSDR, and that resolution of the structures had an adverse effect on the reaction.

The initiation mechanism of iSDR is thought to have some similarities to that of the secondary mode of DNA synthesis of bacteriophage T4 (Magee and Kogoma, 1990). In contrast to the iSDR system, however, endonuclease VII (the product of T4 gene 49), which can recognize recombination intermediates and introduce ss DNA breaks, becomes essential for the secondary mode of DNA synthesis in the case of primase-defective T4 gene 61 mutants (Mosig *et al.*, 1991; see Discussion). In order to confirm the stimulating effect of the *ruvC* mutation observed in our system, the mutant cells were transformed with a derivative of pBR322 (pMZ18-ruvC$^+$) that carried the *ruvC*$^+$ gene under the control of the *lac* promoter. The result shown in Figure 3B indicates that induction of RuvC protein synthesis with isopropyl-β-D-thiogalactopyranoside (IPTG) reduced the iSDR activity in the *ruvC* mutant to the wt level, whereas the pMZ18 vector had no effect. The overproduction of RuvC protein did not affect the level of iSDR in the wt cells

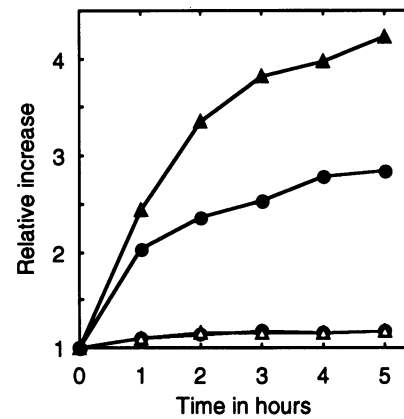

Fig. 4. Effects of a deletion mutation in the *ruvA* and *ruvB* genes on iSDR. HRS1004 (*ruvAB*, ▲△) and TNM554 (*ruvC*, ●○) were grown in the presence of [^{3}H]thymidine (10 μCi/10 μg/ml) and 2′-deoxyadenosine (300 μg/ml) to 1.5 × 10^8 cells/ml. The cells were then treated with NAL as described in Materials and methods. Filled symbols, NAL treated; open symbols, untreated.

(Figure 3B). These results indicate that the enhanced iSDR activity in *ruvC* mutants was due to the loss of the RuvC resolvase activity.

The *recG258*::Tn*10* mutation also enhanced the iSDR activity to a level similar to that in the *ruvC* mutant (Figure 3A). In a strain carrying a deletion that completely removes the *ruvA* and *ruvB* genes (Iwasaki *et al.*, 1989), the iSDR activity was much higher than that in the *ruvC* mutant (Figure 4). These results suggest that branch migration inhibits initiation of iSDR.

Figure 3A also shows that *recD recG* and *recJ recG* double mutants exhibited higher iSDR activity than single mutants, although the effects of the mutations were not additive.

recBC sbcA mutants are inducible for iSDR

Mutations in the *recB* and *recC* genes block induction of iSDR (Magee and Kogoma, 1990). In this study, the iSDR activity was analyzed in a *recB21 recC22 sbcA23* triple mutant (Figure 5). The result shows that the *sbcA23* mutation suppressed the defect in iSDR induction associated with the

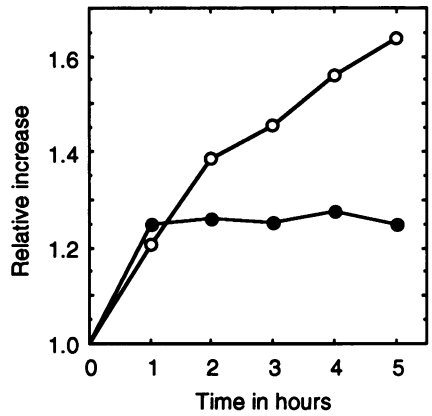

Fig. 5. Effects of the RecE pathway on iSDR. JC8679 (*recBC sbcA*, ○) and JC8691 (*recBC sbcA recE*, ●) were grown in the presence of [^{3}H]thymidine (10 μCi/10 μg/ml) and 2′-deoxyadenosine (300 μg/ml) to 2 × 10^8 cells/ml. The cells were then irradiated with UV at 96 and 12 J/m^2 for JC8679 and JC8691, respectively, and treated as described in Materials and methods. The UV doses were used to achieve approximately equal killing (99.5% for JC8679, 99.4% for JC8691).

recBC mutant genotype. In contrast, a *recB21 recC22 sbcA23 recE159* quadruple mutant did not induce iSDR. Thus, the expression of Exo VIII is required for iSDR in the absence of RecB and RecC.

iSDR produces covalently closed circular (ccc) plasmid DNA molecules

The products of iSDR were analyzed by Southern blot hybridization for minichromosome pOC23 (Messer *et al.*, 1978), which contains one of the origins for iSDR (Magee *et al.*, 1992). Figure 6 shows that the major product of iSDR was ccc DNA monomers. High molecular weight (hmw) plasmid DNA molecules, which migrated with a mobility similar to that of chromosomal DNA fragments, were also observed in the DNA from induced cells, and the amounts of both ccc and hmw DNAs increased during the incubation with RIF and CAM in thymine-starved cells. Some other species of plasmid DNA molecules can also be seen between hmw and ccc molecules for induced samples. Although the structures of these molecules have not yet been determined, the mobility of those molecules suggests that they possibly include ccc multimers, relaxed monomers and linear monomers.

Discussion

RecBCD protein is a multifunctional enzyme (see Introduction). Whereas *recB* and *recC* mutations inhibit iSDR (Magee and Kogoma, 1990), the *recD1903* mutation greatly enhances iSDR (Figure 2). This indicates that some activity of RecBCD is required for iSDR but it is not the exonuclease (Exo V) activity, which the *recD* mutation inactivates. Since *recD* mutants lack the ability to respond to chi sequences (Chaudhury and Smith, 1984; Amundsen *et al.*, 1986), it is unlikely that the chi-sequence specific endonuclease activity of the RecBCD enzyme is involved in initiation of iSDR. This is further supported by the observation that chi-dependent activation of recombination is inhibited in SOS-induced cells (Rinken and Wackernagel, 1992). The ss DNA-specific endonuclease activity of purified

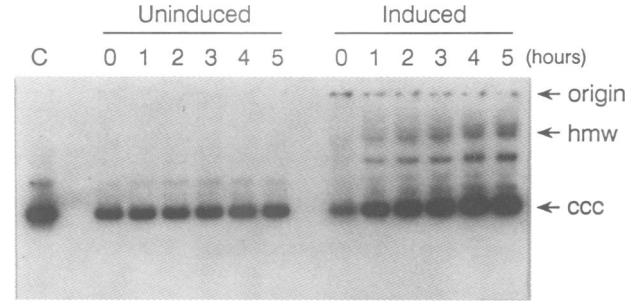

Fig. 6. Analysis of iSDR products. An *oriC* deletion strain (AQ4401) transformed with minichromosome pOC23 was grown in the presence of [^{3}H]thymine (5 μCi/8 μg/ml) to 2 × 10^8 cells/ml and was starved of thymine for 120 min (induced). A mixture of thymine, RIF and CAM was added at the end of starvation (time 0), and samples (0.5 ml) were withdrawn at intervals. For a control (uninduced), the mixture was added without starvation. Total DNA was prepared from each sample, and the amounts of labeled DNA normalized to give equal ^{3}H counts were loaded on a 0.8% agarose gel. After electrophoresis, the gel was treated with 0.25 M HCl for 30 min and DNA was capillary-blotted on to a nylon membrane with 0.4 M NaOH (Magee *et al.*, 1992). The resulting Southern blot was probed with a ^{32}P-labeled DNA fragment carrying a sequence (752 bp) between the *Pst*I and *Eco*RI sites of pBR322 containing a part of the *bla* gene (Sutcliff, 1978). For a size marker, a sample of pOC23 ccc DNA purified with a column (Qiagen, Chatsworth, CA) was loaded in lane C. ccc, covalently closed circular monomers; hmw, high molecular weight molecules; origin, the origin of electrophoresis migration.

RecBC enzyme is much lower than that of purified RecBCD holoenzyme (Palas and Kushner, 1990). Thus, it is also unlikely that this activity is necessary for initiation of iSDR. On the other hand, requirement of the recombinase activity of RecA for iSDR (Figure 1) strongly suggests that the helicase activity of RecBCD is essential for iSDR; unwinding of the duplex would produce ss DNA substrates for RecA protein.

Based on the results presented in this and previous reports (Magee and Kogoma, 1990; Magee *et al.*, 1992), we wish to propose a model for the initiation mechanism of iSDR (Figure 7). We hypothesize that upon the SOS response some protein(s) is induced, which can recognize the origin for iSDR on a ccc DNA and introduce a double-strand break within or near the origin, generating a linear ds DNA. The RecBCD enzyme bound to the end of the linear DNA would unwind the duplex and produce ss DNA ends (step A). In *recBC sbcA* mutant cells, RecE protein (Exo VIII) could produce ss DNA ends by digesting one strand of ds DNA from the 5′ end. The ss DNA with a 3′-OH end would then invade a homologous region of another circular DNA molecule by the action of RecA protein, generating a D-loop structure (step B; Smith, 1988; Viret *et al.*, 1991). Replication of the circular molecule could be initiated using the invading 3′-OH end as a primer (Formosa and Alberts, 1986). For a net increase of DNA molecules by this pathway, it might be necessary that both ends of the linear DNA are used for the priming of DNA synthesis. Subsequently, endonuclease cleavage would resolve the D-loop structure (step C). Since the major product of the replication is ccc monomers in wt cells (Figure 6), it is most likely that continuation of the leading strand synthesis followed by the lagging strand synthesis on the displaced ss DNA would enlarge the bubble structure and result in the formation of two identical ccc DNA molecules (step D). This is consistent

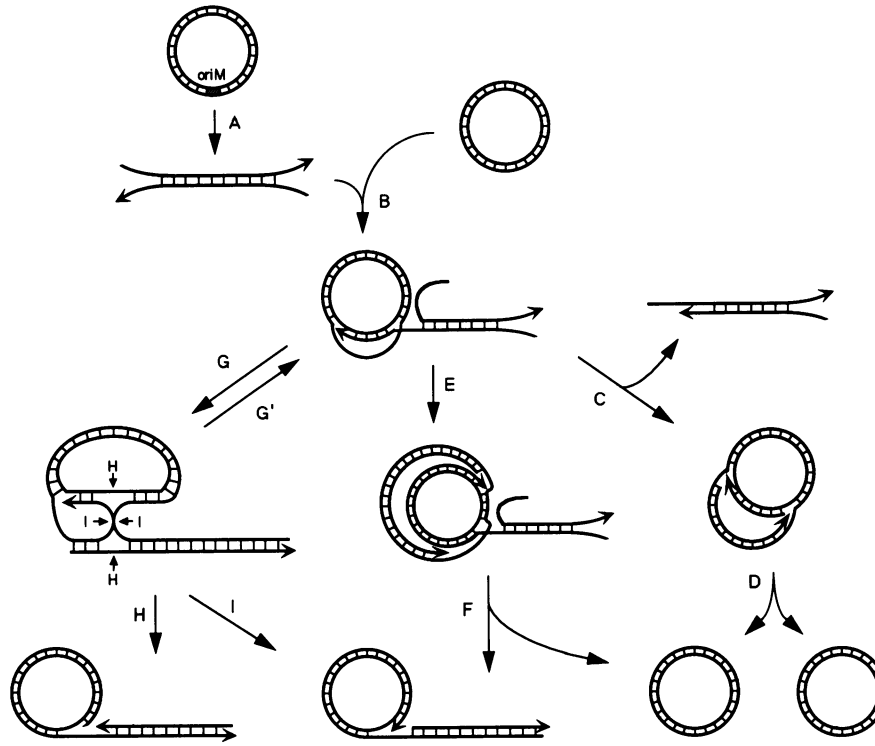

Fig. 7. A model for the initiation mechanism of iSDR. Arrow heads represent the 3′ termini of DNA strands. Small arrows labeled with letters at the Holliday junction point out the cutting sites by resolvase for the corresponding steps. Supercoiling of circular molecules is ignored. See text for details.

with the result that iSDR is a semi-conservative mode of DNA synthesis (Kogoma and Lark, 1970). In the absence of the Exo V activity of RecBCD (i.e. in *recD* mutants), a template for rolling-circle replication might be created from the D-loop structure after one round of replication primed by the invading ss DNA (steps E and F).

According to this model, inactivation of the helicase activity of RecBCD by *recB* and *recC* mutations would inhibit iSDR in the initiation step, unless Exo VIII is induced by an *sbcA* mutation. Inactivation of the recombination activity of RecA protein would also result in loss of the iSDR activity. On the other hand, inactivation of the Exo V activity of RecBCD by *recD* mutations would protect the invading ss DNA from degradation and result in an increase in the amount of the D-loop structure, which leads to the enhancement of iSDR. Similar enhancement of iSDR by inactivation of DNA polymerase I has recently been reported (Ruscitti and Linn, 1992). This effect could also be explained by the protection of ss DNA from degradation by the ss DNA-specific exonuclease activity (exonuclease II) of DNA polymerase I (Kornberg and Baker, 1992). In *recD* mutant cells, rolling-circle replication might also contribute to the enhancement of iSDR (see below). It should be noted that after SOS induction the Exo V activity of RecBCD is inhibited by inducible proteins (Pollard and Randall, 1973; Marsden *et al.*, 1974; Kannan and Dharmalingam, 1990). This would favor the effective initiation of iSDR by protecting ss DNA in induced $recD^+$ cells.

Enhancement of iSDR was also observed in the *recJ284* mutant (Figure 2A). In addition to the RecBCD enzyme, exonuclease III (3′ → 5′; Kornberg and Baker, 1992) followed by RecJ exonuclease (5′ → 3′; Lovett and Kolodner, 1989) would contribute to degradation of the template for rolling-circle replication formed by steps F and I (see below) if the initial double-strand break created blunt or recessed 3′ termini, which are recognized by Exo III. Thus the protection of the template from degradation may be the reason for the weak enhancement of iSDR in the *recJ* mutant. Since cellular functions of RecF and RecN proteins have not been elucidated, the reason for a slight reduction of the iSDR activity in the *recF332* and *recN261* mutants (Figure 2A) is at present not understood.

Once a D-loop structure is created, a DNA molecule containing a Holliday junction would then be generated (step G). If branch migration is prevented at this stage, a reverse process (step G′) would regenerate a D-loop structure which is used for initiation of iSDR. This could be a reason for the enhanced iSDR in *recG* and *ruvAB* mutants. The enhancing effect is higher in *ruvAB* mutants than in *recG* mutants (compare Figures 3A and 4). Since the expression of the *ruvAB* genes is under the control of LexA, the increased synthesis of RuvAB following SOS induction (Shurvinton and Lloyd, 1982) would activate branch migration and reduce the amount of D-loops efficiently in *recG* mutants. Resolution of the junction (step H or I) leads to the formation of one of the two types of templates for rolling-circle replication depending on the cutting sites. In the presence of the Exo V activity of RecBCD, these molecules could be degraded (Cohen and Clark, 1986; Silberstein and Cohen, 1987; Niki *et al.*, 1990). Thus, prevention of the resolution in *ruvC* (and possibly in *recG*) mutants (Lloyd, 1991) would also result in an increase in the amount of the D-loop structure available for iSDR initiation.

Late in the infection cycle of bacteriophage T4, initiation of phage DNA replication becomes dependent on gene functions that are involved in genetic recombination (Luder and Mosig, 1982; Kozinski, 1983; Kreuzer *et al.*, 1988).

In this mode of DNA synthesis, ss DNA at the end of the T4 genome, which is created by the primary mode of DNA synthesis, is proposed to invade a homologous region of another or the same T4 DNA molecule by the action of UvsX protein (formation of a D-loop structure) and to prime the leading strand synthesis. Priming of the lagging strand synthesis is thought to occur on the displaced ss DNA of the D-loop by the product of T4 gene 61 (primase). However, primase-defective T4 mutants are viable. In these mutants, functional Endo VII (the product of T4 gene 49) was found to be required for the lagging strand synthesis (Mosig *et al.*, 1991). Thus, it is proposed that Endo VII introduces a ss DNA break at the D-loop structure and generates a 3′ end that is used to prime DNA synthesis on the displaced ss DNA. It is, therefore, of great interest to analyze the iSDR activity in *E.coli priA* mutants which are thought to have a defect in primer synthesis for lagging strands.

Duplex opening is a prerequisite for initiation of replication of ds DNA molecules. In the event of initiation at *oriC*, DnaA protein is known to play a key role in duplex unwinding (Bramhill and Kornberg, 1988). Transcription that is essential for initiation at *oriC* is also proposed to help this step by altering superhelical structure of *oriC* DNA (Baker and Kornberg, 1988; Asai *et al.*, 1990; Skarstad *et al.*, 1990). In *rnhA* mutants which lack RNase HI activity, DNA replication is known to occur at *oriK*s (Kogoma, 1986). In this case, formation of persisting RNA−DNA hybrids (R-loops) is thought to be responsible for duplex opening (von Meyenburg *et al.*, 1987). In this report, we have proposed that duplex opening for iSDR initiation could be achieved by D-loop formation. This model accounts for the independence of iSDR from the normally required DnaA protein, transcription and protein synthesis for initiation. There are, however, a number of unanswered questions. For example, what is the nature of the inducible factor which triggers the initiation reaction? How is lagging strand DNA synthesis initiated? Why is the activated form of RecA protein required? We have recently localized the origin activity in two domains within the minimal *oriC* sequence (T.Asai and T.Kogoma, manuscript in preparation). These domains are known to bind with high affinity to outer membrane preparations (Kusano *et al.*, 1984). Further characterization of the nucleotide sequences and additional factors required for iSDR initiation is expected to help answer these and other questions about this intriguing DNA replication system.

Materials and methods

Media and growth conditions

Unless otherwise stated, cells were grown at 37°C, with aeration by shaking, in M9 salts−glucose medium supplemented with casamino acids (0.2%;

Table II. *Escherichia coli* K-12 strains

Strain	Relevant genotype[a,b,c,d,e]	Reference/construction
AQ634[a]	*rec*$^+$ *thyA deoB* (or *C*)	Ogawa *et al.*, 1984
AQ3359[a]	*recN261 tyrA*::Tn*10 thyA deoB* (*C*)	AQ634 × P1. SP215 select Tc^r, MMC^s
AQ3362[a]	*recJ284*::Tn*10 thyA deoB* (*C*)	AQ634 × P1. JC12123 select Tc^r, UV^s
AQ3788	*oriCdel-1071* (Tc^r) *zif90*::pML*31*(Km^r)	Kogoma and Kline, 1987
AQ4241[b]	*rec*$^+$ *thyA deoB* (*C*)	Magee and Kogoma, 1991
AQ4382[b]	*rec*$^+$ *thyA deoB* (*C*) *zif90*::pML*31*	AQ4241 × P1. AQ3788 select Km^r
AQ4401[b]	*rec*$^+$ *thyA deoB* (*C*) *zif90*::pML*31 oriCdel-1071*	AQ4382 × P1. AQ3788 select Tc^r
AQ4790[a]	*recF332*::Tn*3 thyA deoB* (or *C*)	AQ634 × P1. JC10990 select Ap^r
AQ7960[a]	*ruvC51 eda-51*::Tn*10 thyA deoB* (*C*)	AQ634 × P1. TNM554 select Tc^r, UV^s
AQ8014[c]	*recAdel-306 thyA deoB* (*C*)	*thyA deoB* (or *C*) of GY7313
AQ8022[a]	*recJ284*::dTn*10* (Tc^s) *thyA deoB* (*C*)	AQ3362 select Tc^s on Bochner plates
AQ8070[a]	*recJ284*::dTn*10 recD1903*::mini-*tet thyA deoB* (*C*)	AQ8022 × P1. DPB271 select Tc^r, T4.2^s
AQ8110[c]	AQ8014 transformed with miniF-*recA428*	
AQ8112[c]	AQ8014 transformed with miniF-*recA730*	
AQ8115[c]	AQ8014 transformed with miniF-*recA*$^+$	
AQ8130[a]	*recG258*::Tn*10kan thyA deoB* (*C*)	AQ634 × P1. N2731 select Km^r, UV^s
AQ8134[a]	*recD1903*::mini-*tet thyA deoB* (*C*)	AQ634 × P1. AQ8070 select Tc^r, T4.2^s
AQ8194[a]	*recJ284*::dTn*10 recG258*::Tn*10kan thyA deoB* (*C*)	AQ8022 × P1. N2731 select Km^r
AQ8208[a]	*recD1903*::mini-*tet recG258*::Tn*10kan thyA deoB* (*C*)	AQ8134 × P1. N2731 select Km^r, UV^s
DPB271	*recD1903*::mini-*tet*	Biek and Cohen, 1986
GY7066[e]	*lacMS286* Φ80*dIIlacBK1* Δ*recA306*	Dutreix *et al.*, 1989
GY7236[f]	Hfr J2 *leu*::Tn*9*	Dutreix *et al.*, 1989
GY7313[c]	λ*sfiA*::*lacZ*$^+$ Δ*lac sfiA211* Δ*recA306*	Bailone *et al.*, 1988
HRS1004[d]	Δ*ruvA* Δ*ruvB*	Iwasaki *et al.*, 1989
JC8679[d]	*recB21 recC22 sbcA23*	A.J.Clark
JC8691[d]	*recB21 recC22 sbcA23 recE159*	A.J.Clark
JC10990	*recF332*::Tn*3*	A.J.Clark
JC12123	*recJ284*::Tn*10*	Lovett and Clark, 1984
N2731	*recG258*::Tn*10kan*	Lloyd and Buckman, 1991a
SP215	*recN261 tyrA16*::Tn*10*	Picksley *et al.*, 1984
TNM554[d]	*ruvC51 eda-51*::Tn*10*	G.J.Sharples

The remaining genotypes are: a, F$^-$ *ilv metB his-29 trpA9605 pro*; b, F$^-$ *trp his metE thi ara lacZ galK mtl rbs*$^+$ *rpsL tonA* T6^r *sup-38*; c, *thr-1 leuB6 his-4 argE3 ilv*^{ts} Δ*lac* λp[*sfiA*::*lacZ*$^+$ cI (Ind$^-$)] *sfiA211 galK2 ara-15 xyl-15 mtl-1 srl*::Tn*10 tsx-33 rpsL31 supE44*; d, F$^-$ *argE3 his-4 leu-6 proA2 thr-1 thi-1 rpsL31 galK2 lacY1 ara-14 xyl-5 mtl-1 supE44*; e, *metB pyrE thi rpsL*; f, *lacZ*::Tn*5*. MMC^s, sensitivity to mitomycin C; T4.2^s, sensitivity to T4 gene 2 mutant phage; UV^s, sensitivity to UV.

Difco Laboratories, Detroit, MI), required amino acids (50 μg/ml), thymine (8 μg/ml) and thiamine hydrochloride (2 μg/ml). LBT and LAT broth have been described previously (Devoret *et al.*, 1983). For the selection of antibiotic-resistant *E.coli* cells, antibiotics were added at the following concentrations: ampicillin (40 μg/ml), kanamycin (25 μg/ml), tetracycline (20 μg/ml), streptomycin (100 μg/ml) and chloramphenicol (25 μg/ml).

Chemicals and radioisotopes

Chemicals were purchased from Sigma Chemical Co. (St Louis, MO). [methyl-^{3}H]thymine and [α-^{32}P]deoxycytidine-5′-triphosphate were from New England Nuclear Corp. (Boston, MA).

E.coli strains and plasmids

The *E.coli* strains are listed in Table II. Strains were constructed by phage P1-mediated transduction. The presence of the *recD1903*::mini-*tet* mutation was confirmed by the sensitivity to T4 gene 2 mutant phage. The presence of the *recJ284*::Tn*10*, *ruvC51* and *recG258*::Tn*10kan* mutations was confirmed by sensitivity to UV irradiation. The *recN261* mutation was verified by sensitivity to mitomycin C. The *recN* and *ruvC* mutations were transduced by virtue of their linkage to the *tyrA*::Tn*10* and *eda-51*::Tn*10* mutations, respectively.

The *recA730* mutation was transferred from JM30 (Witkin and Kogoma, 1984) to pGY5887 (see below) by *in vivo* recombination. AQ8014 was constructed from GY7313 by selecting thymine-requiring cells after trimethoprim treatment (Miller, 1972).

Plasmid pMZ18-ruvC$^+$ was constructed by inserting the *Eco*RI−*Hin*dIII fragment of pGS762 (Sharples and Lloyd, 1991), which contains the *ruvC*$^+$ gene, into the multiple cloning site of pMZ18 between the *Eco*RI and *Hin*dIII sites downstream from the *lac* promoter. pMZ18 was constructed by M.Chambers in this laboratory from a derivative of pBR322 (pTKQ27; Kogoma, 1984) which carries a *Hae*II(232)−*Hae*II(2347) deletion. The *Eco*RI(4359)−*Hin*dIII(29) fragment (2215 bp) of pTKQ27 was prepared and both ends of the fragment were filled with Klenow fragment. The fragment was then ligated to the *Hae*II fragment (445 bp) of pUC18, which had been treated with mung bean nuclease, to construct pMZ18.

Isolation of recA mutants

MiniF plasmid pMF21 (Manis and Kline, 1977) carrying the *recA*$^+$ gene (pGY5887) was mutagenized by treatment with hydroxylamine and introduced into GY7313 cells. Since the host cells carried an *sfiA*::*lacZ* operon fusion, protease-constitutive *recA* mutants could be detected as blue colonies on M9 plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside. The plasmids were isolated from blue colonies and introduced into GY7066 cells to measure the recombination activity of mutated RecA proteins.

Assay for RecA recombinase activity

Three different assays were employed to examine the recombinase activity in *recA* mutants. GY7066 cells were transformed with a miniF plasmid carrying the *recA*$^+$ or the *recA428* gene, or with the vector plasmid (Δ*recA*).

(i) Conjugational recombination. Cells carrying miniF were grown in LBT broth to 10^8 cells/ml and mated with Hfr strain GY7236 for 30 min. The ex-conjugants were scored for Cm^r and Sm^r on LAT plates containing chloramphenicol and streptomycin.

(ii) Intrachromosomal recombination. GY7066 carries two copies of the *lac* operon with non-overlapping deletions (Konrad and Lehman, 1975). The number of Lac$^+$ papillae resulting from recombination between the two defective *lac* genes was determined as described previously (Dutreix *et al.*, 1989).

(iii) Plating efficiency of λ*bio*. Formation of packageable multimers of λ*bio* DNA is dependent on the recombination function of RecA (Zissler *et al.*, 1971), since the *bio10* mutation removes recombination genes of λ phage (Court and Oppenheim, 1983).

Determination of the iSDR activity

A method to determine the iSDR activity was as described previously (Magee and Kogoma, 1990) with a minor modification. In the previous experiments, the amount of [^{3}H]thymine incorporated per cell was employed to show the iSDR activity. However, we noticed that this value did not represent the precise iSDR activity in some mutants analyzed in this study. This is because the number of chromosomes (i.e. the number of origins for iSDR) per cell in some mutants is different from that in the wt. Thus, experiments were carried out as follows. Cells were grown to exponential phase in the presence of [^{3}H]thymine (10 μCi/8 μg/ml) for three generations. Radioactive thymine was then removed by filtration and the cells were resuspended in medium lacking required thymine. Immediately, a sample (90 μl) was withdrawn to determine the radioactivity of the trichloroacetic acid (TCA)-insoluble fraction (the initial radioactivity). The cell suspension was then split into two halves and a mixture of [^{3}H]thymine (10 μCi/8 μg/ml), RIF (200 μg/ml) and CAM (150 μg/ml) was immediately added to one half of the suspension (uninduced control). The other half of the suspension was starved of thymine for a duration approximately equivalent to two doublings (shown in the figure legends) and then the mixture was added. Samples (90 μl) were taken at the indicated times and the radioactivity of the TCA-insoluble fraction of each sample was determined by liquid scintillation counting. The relative increase of DNA during iSDR was determined by dividing the radioactivity of each sample taken after the addition of RIF and CAM by the initial radioactivity.

In *thyA*$^+$ strains, the induction of iSDR was achieved by treating cells with nalidixic acid (NAL). Cells were grown in the presence of [^{3}H]thymidine (10 μCi/10 μg/ml) and 2′-deoxyadenosine (300 μg/ml). After removing [^{3}H]thymidine by filtration, cells were suspended in medium containing NAL (50 μg/ml). After taking a sample to determine the initial radioactivity, one half of the suspension (uninduced control) was filtered immediately to remove NAL, and resuspended in medium containing a mixture of [^{3}H]thymidine (10 μCi/10 μg/ml), 2′-deoxyadenosine (300 μg/ml), CAM (150 μg/ml) and RIF (200 μg/ml). The other half of the suspension was incubated at 37°C for 70 min. Then the cells were filtered and suspended in medium containing the mixture.

For induction by UV irradiation, cells grown to exponential phase in the presence of [^{3}H]thymidine and 2′-deoxyadenosine were filtered and resuspended in M9 buffer. After taking a sample for the initial radioactivity, the cells were irradiated with UV. The cell suspension was then supplemented with glucose, casamino acids, thiamine hydrochloride and the required amino acids. An aliquot was taken immediately after UV irradiation, diluted and plated to determine survival (Ruscitti and Linn, 1992). After 60 min of growth, a mixture of [^{3}H]thymidine, 2′-deoxyadenosine, CAM and RIF was added.

Extraction of the chromosomal DNA and Southern blot hybridization

These were carried out as described previously (Magee *et al.*, 1992). The Random Primers DNA Labeling System (BRL, Gaithersburg, MD) was used to obtain ^{32}P-labeled DNA probes with a high specific activity (~2 × 10^9 c.p.m./μg DNA).

Determination of sfiA::lacZ expression

This was carried out as described previously (Kogoma *et al.*, 1993).

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